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Ambient orchard volatiles from California almonds

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ABSTRACT

The volatile emissions of various plant parts of almonds have been studied via various techniques in the past. These analyses have typically been performed on single cultivars and hence may not be representative of the volatiles found in an entire almond orchard. Recent reports suggest some almond volatiles exhibit semiochemical activities for the navel orangeworm (NOW), a major insect pest of almonds; thus, the volatile composition of the comprehensive almond orchard would be helpful to research concerning NOW. The ambient volatile emissions of an almond orchard containing the cultivar Nonpareil and associated pollenizers were collected at four intervals during the 2009 growing season from orchards in the south Central Valley of California. The volatiles hexanal, octanal, nonanal, benzaldehyde, acetophenone, ethyl benzoate, methyl salicylate, and phenol were consistent in their presence and in relatively high amounts. The orchard volatile composition was analyzed via electroantennogram (EAG) analysis, which produced strong responses from NOW antennae.
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1. Introduction

The Central Valley of California is the world's top producer of almonds, *Prunus dulcis* (P. Mill) D.A. Webb, with 710,000 bearing acres (USDA-NASS, 2010) and supplies nearly 80% of the world's almond demands. The Nonpareil almond variety represents the most widely planted cultivar in the Central Valley and comprises ca. 37% of the total acres of varieties grown. Other cultivars and pollenizers such as Butte, Carmel, Padre, Sonora, Monterey, and Aldrich combined comprise ca. 54% of the total almond acreage (USDA-NASS, 2009).

The volatile emissions of almonds and corresponding plant parts have been investigated with reports on the steam distillation of dried almond hulls (Buttery et al., 1980), *ex situ* whole damaged and undamaged almonds (Beck et al., 2008), and the *in situ* emission of Nonpareil almonds over a growing season (Beck et al., 2009), among others. However, little is known regarding the general atmospheric bouquet emitted from an orchard and what affect the orchard bouquet may have on host-plant locating behavior of insects.

The navel orangeworm (NOW), *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is a major insect pest of California tree nuts, almonds in particular. Feeding damage by NOW larvae reduces nut kernel quality resulting in wide-spread economic loss to the almond industry. In addition to the direct feeding damage, NOW larvae have been shown to contribute to contamination by *Aspergillus flavus*, a ubiquitous fungus of tree nut orchards that is capable of producing aflatoxins, which represent a serious food safety problem due to their carcinogenic and teratogenic attributes (Campbell et al., 2003).

Host-plant location by an insect is in part dependent upon its ability to detect specific semiochemicals, and a complex mixture of ubiquitous plant volatiles may be necessary to elicit an appropriate response from insects to their host-plant (Bruce et al., 2005).

The goals of this investigation were to: collect the ambient volatile emission of almond orchards from the southern Central Valley of California during a typical growing season (ca. April through August); identify the major volatiles emitted and their relative quantities; and, determine the general chemoreceptivity of female NOW adult moths to the collected volatile bouquet.

2. Results and discussion

A total of 25 volatiles were collected in minor to major amounts, separated via GC–MS, and identified (Table 1). Once desorbed, the volatiles were quantified using an internal standard and the relative amount of each was calculated. Of the 25 principal volatiles collected from the almond orchards, eight were consistent throughout the spring to summer collection periods, and in relatively high (>20 ng m^{-3}) amounts: hexanal, octanal, nonanal, benzaldehyde, acetophenone, ethyl benzoate, methyl salicylate, and phenol. A number of other identified volatiles were either

Abbreviations: EAG, electroantennogram; NOW, navel orangeworm; RIs, retention indices.

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Table 1
Ambient almond orchard volatile amounts from Kern County, California collected during the 2009 growing season.

#	Compound ID	DB-Wax[a]			Ambient almond				Avg	s.e.
		RT	RI		Volatile amounts (ng m^{-3})[b]					
			Calc'd	Lit	Collection 1	Collection 2	Collection 3	Collection 4		
1	Hexanal	6.49	1077	1077	26.8	49.3	31.1	23.3	32.6	5.8
2	Undecane	6.76	1088	1100	0.0	0.0	0.0	7.0	1.8	1.8
3	Cumene	8.77	1167	1168	3.0	5.3	0.0	3.5	3.0	1.1
4	Heptanal	9.11	1180	1180	12.2	13.1	11.4	13.1	12.4	0.4
5	Limonene	9.48	1194	1195	3.7	0.6	0.0	0.0	1.1	0.9
6	p-Cymene	11.59	1266	1264	1.8	4.8	4.5	6.9	4.5	1.0
7	Octanal	12.17	1285	1284	78.1	108.2	49.6	50.1	71.5	13.9
8	Nonanal	15.42	1390	1389	237.4	338.4	161.2	169.4	226.6	41.0
9	Acetic acid	17.29	1451	1447	11.1	3.9	13.9	11.2	10.0	2.1
10	Decanal	18.65	1495	1495	3.8	0.0	18.1	21.7	10.9	5.3
11	Benzaldehyde	19.20	1515	1516	306.8	165.3	306.6	1971.5	687.5	429.3
12	Benzonitrile	21.63	1595	1597	3.9	1.7	3.5	10.0	4.8	1.8
13	γ-Pentanolactone	21.78	1601	1600	3.4	8.1	10.8	6.2	7.1	1.5
14	Methyl benzoate	22.19	1615	1616	7.7	9.3	14.8	7.0	9.7	1.8
15	Sabina ketone[c]	22.51	1626	n/a	12.6	0.0	5.3	3.2	5.3	2.7
16	Phenylacetaldehyde	22.70	1633	1636	11.9	10.4	19.0	25.9	16.8	3.6
17	Acetophenone	22.98	1642	1645	151.5	224.8	263.8	355.4	248.9	42.5
18	Ethyl benzoate	23.51	1661	1661	51.9	59.7	23.3	31.8	41.7	8.5
19	Salicylaldehyde	23.72	1668	1673	5.4	7.2	5.3	9.8	6.9	1.1
20	γ-Hexanolactone	24.39	1691	1699	4.3	9.6	13.9	13.5	10.3	2.2
21	Naphthalene	25.44	1730	1734	0.0	0.0	1.4	3.0	1.1	0.7
22	Methyl salicylate	26.46	1767	1771	122.7	191.7	76.7	77.5	117.2	27.1
23	1-Methylnaphthalene	29.35	1876	1884	0.0	11.8	14.8	0.0	6.6	3.9
24	Phenol	32.48	2002	2000	74.7	83.7	74.4	87.8	80.2	3.4
25	p-Anisaldehyde	32.84	2017	2024	3.5	0.0	0.0	11.3	3.7	2.7
	Collection dates				4/23–5/5	6/30–7/7	7/7–7/15	8/11–8/21		
	Relative nut phenology				kernel filling	hull split[d]	hull split[d,e]	hull split[f]		

[a] RI calculated relative to *n*-alkanes on DB-Wax and compared to literature and internally generated data base values.
[b] Ambient volatile amount calculated using total analyzed relative amount of each volatile per volume of air collected (total number of minutes × flow rate for each Tenax cartridge).
[c] Tentative assignment, compound not available for authentication.
[d] Primarily relative to Nonpareil.
[e] Start of hull split for pollenizers.
[f] Primarily relative to pollenizers, late for Nonpareil.

transient and/or at a very low relative concentration within the ambient orchard environment. The average values (Avg) shown in Table 1 provide a quick reference for relatively high amount of volatiles (a large Avg value), and the standard error (s.e.) describes either consistent or transient emission over the four collections. For instance, a low s.e. may indicate consistent emission of that volatile (*e.g.*, heptanal with an average emission of 12.4 ± 0.4 ng m^{-3}); whereas a larger s.e. may indicate either an upward or downward trend in volatile emission (*e.g.*, phenylacetaldehyde, which increases over time, with an average emission of 16.8 ± 3.6 ng m^{-3}). It should be noted that alkyl aromatics were also detected and were identified components from orchard maintenance pesticide sprays; however, these residual volatile amounts were not evaluated for this report.

Benzaldehyde, a ubiquitous plant volatile known as a primary component of bitter almond oil (Arctander, 1960), was detected as the most prevalent volatile with a range of 165–1972 ng m^{-3}. Benzaldehyde, as well as all of the aldehydes, was detected as both the aldehyde and the corresponding acid. This is presumably due to air oxidation of the aldehydes while absorbed on the Tenax medium. To verify this assumption, the aldehydes detected in this study were loaded onto a cartridge of Tenax and placed in an oven at 38 °C with airflow of 4 l min^{-1}. The components were desorbed after one week and the corresponding acids were detected in varying amounts. Thus, the aldehyde amounts shown in Table 1 are understood to be a combination of both the aldehyde and acid form, and include the relative amounts for their detected associated acids.

The C_6–C_{10} alkyl aldehydes, of which hexanal, octanal, and nonanal were consistently detected and in relatively large amounts, along with lesser amounts for heptanal and decanal, are known as fatty acid breakdown products (Frankel, 1982). Nonanal, the volatile with the third highest presence, has been detected in other almond volatile investigations (Buttery et al., 1980; Beck et al., 2008, 2009), yet the studies by Beck et al. did not report finding the other alkyl aldehydes shown in Table 1. The presence of the C_6–C_{10} alkyl aldehydes in a previous report by Buttery et al. bring to question the specific reason for presence and/or increased emission of this class of compounds. It should also be emphasized that the ambient volatiles collected during this study may be representative of what insects encounter while present in the orchards, and are not necessarily only from the almond tissues, but may also originate from soil, microbes, and/or weeds. The volatiles noted earlier from orchard maintenance sprays provide a good example of other orchard content odors.

Another consistent and major volatile was acetophenone followed by other aryl compounds with moderate volatile amounts—ethyl benzoate, methyl salicylate, and phenol. Acetophenone, a ubiquitous volatile from several plant families (El-Sayed, 2010), showed a progressive increase in ambient volatile presence (Table 1) over the growing season. Ethyl benzoate is a ubiquitous volatile emitted from numerous plants (El-Sayed, 2010), including almonds (Beck et al., 2008). Ethyl benzoate has been reported as possessing some ability to attract NOW, in addition to the structurally similar methyl benzoate (Price et al., 1967), a minor but consistent volatile in this study. Similarly, the ubiquitous plant volatile methyl salicylate has demonstrated semiochemical activity (El-Sayed, 2010) for a number of species. A surprising volatile detected was phenol, for which this would be the first report of its detection in almonds. Though this report does

not purport to pinpoint the exact origin of phenol emission, it should be noted that it was detected during our *in situ* almond study but was not reported at that time due to its transient nature.

One important finding of this investigation was the quantified changes in relative volatile emissions over the spring to summer collection period. For example, acetophenone showed a steady and gradual increase in volatile amount over the course of collections. Nonanal was detected in greater relative quantities in the first two collections, and then decreased as acetophenone increased in the later collections. A previous study (Beck et al., 2009) evaluated the *in situ* volatile emission of almonds from just the cultivar Nonpareil via 1 h SPME collections at ca. one week intervals and over the course of a growing season. However, that experimental design did not assess the volatile bouquet composition of the whole orchard—the orchard volatiles that are most likely encountered by insects during orchard flight. An additional contrast between the present study and the *in situ* investigation is the disparity of volatiles identified. For instance, the *in situ* investigation identified numerous sesquiterpenes, a class of compounds not detected in the present study, yet have been detected by other Tenax collection experiments on almonds (Beck et al., 2008). This phenomenon may be explained by the volatile "snapshot" analyzed in the *in situ* investigation by use of SPME at a specific time during the day, versus the present study that collected volatiles continuously (24 h/day) for several days. A change in emission as a function of time of day (diurnal emissions) is a well-established phenomenon (Casado et al., 2008). Additionally, the amounts of sesquiterpenes detected *in situ* may be dilute relative to other major volatile emissions, thus below the level of detection for the volatile samples collected. This issue needs to be addressed further, and may be investigated by the use of the volatile collection system modified with a timer and two cartridges for separate night and day volatile collections.

The change in emission patterns for some of the compounds over the course of volatile collections is suggestive of a dynamic versus static volatile medium encountered by insects, or their progressive generations, throughout the growing season. However, it should be recalled that some of these volatiles may have origins other than the almond trees and could be a result of orchard soil maintenance (fertilizers, sprays, mowing, etc.). To be prudent, these influences can be taken into consideration when analyzing the ambient orchard emissions and what the insect is encountering. To delineate volatile origin, subsequent studies would have to factor in concurrent volatile analyses of the soil, leaves, and fruit in the surrounding collection area. The most obvious example of this dynamic emission was the change in relative volatile amounts of acetophenone, which showed an increase in emission over time, and nonanal which initially increased, but then dropped off in the last two collection periods. Whether these changes are linked to specific nut phenological stages, and how such dynamics in the volatile medium potentially affects NOW behavior could be considered in future studies. It should be noted that a prototype of the collection system was used during portions of the 2008 growing season and in the same orchards. Though there were issues with flow control and constant collection periods, the preliminary results (unpublished) from the prototype were consistent in terms of volatiles collected and relative ratios when compared to the results obtained in 2009, and reported here.

Finally, to assess the overall influence these orchard emissions may have on NOW, electroantennogram (EAG) experiments were performed on aliquots of the collected natural bouquet of volatiles. The EAG antennal recordings of female NOW to the collected ambient almond orchard volatile bouquet ($n = 2$) indicated relatively strong electrophysiological response to this complex and natural volatile medium. Owing to the limited total amounts of collected volatiles there was just enough material remaining after GC–MS analyses to determine the EAG response of female NOW toward the total bouquet of volatiles naturally encountered by NOW moths for only collections made in early May (Collection 1) and early July (Collection 2). The female NOW antennal response to Collection 1 was 947 μV (842 μV corrected) and 1026 μV (895 μV corrected) to Collection 2. The relatively high μV amplitude antennal responses to the two bouquets demonstrate a chemoreceptive sensitivity to these background ambient volatiles of the almond orchards, but do not necessarily indicate a behavioral response. For comparison, the average male EAG response to the female sex pheromone major aldehyde component was ca. 1200 μV using the identical setup as described above.

The goals of the investigation were successfully realized—a collection system that allowed for the monitoring of the atmospheric volatiles in an orchard was utilized and the ambient almond orchard volatiles were collected, identified, and relatively quantified. Additionally, the female NOW antennae demonstrated general chemoreceptivity to the collected volatile bouquet. The results of this investigation provide evidence for the potential role of some or a number of ambient almond orchard volatiles as potential semiochemicals for NOW; but, more importantly provide a starting point for in-depth electrophysiological and bioassay experiments of the individual volatiles. Continued research with increased number of volatile collections during the growing season will provide higher resolution for detection of changes in relative volatile amounts over the phenological maturation of almonds. This higher resolution may reveal discrete volatile dynamics throughout the season that in turn may lead to more insights regarding orchard volatiles and how they are perceived by NOW.

3. Experimental

3.1. Orchard

The collection site in the southern Central Valley was located near Lost Hills, CA (Kern County) on the property of Paramount Farming Company. The plot, ca. 160 acres, contained the almond varieties Nonpareil, Carmel, and Monterey in a 2:1:1 ratio, and was contiguous to ca. 881 acres of Butte and Padre (1:1) varieties to the East (upwind). The plot containing the noted varieties was chosen based on three criteria: (1) common varieties—the most common almond varieties found in California orchards are Nonpareil (37% of total acres), Carmel (13%), Butte (12%), and Monterey (10%) (Almond Board, 2010); (2) location relative to other commodities; and (3) the largest plot size we could find that fit the first two criteria. The last two criteria were important to minimize the collection of volatiles from non-almond commodities. A total of two ambient collections ($n = 2$) were performed per experiment. The two collection boxes were placed deep within each plot in the tree rows of Nonpareil, spaced ca. 85 m apart in the north/south line, and ca. 100 m to the closest orchard edge on the west side.

3.2. Volatile collections

Volatiles were absorbed onto Tenax via a large-scale volatile collection system comprised of the following: glass cartridges containing Tenax (10 g, 2.5 cm × 15 cm) fastened inside of a closed PVC cylinder with a port for vacuum attachment and a screened port open to ambient orchard air; the Tenax cartridge was attached via Teflon 0.64 cm tubing to a 12 VDC eccentric diaphragm pump (Schwarzer Precision, Germany) powered by a high-capacity battery (AGM-92AH, West Marine, Richmond, CA), and charged by an 18 VDC, 1.8 Amp solar panel (PowerUp, Baltimore, MD). The vacuum pump, electronic controller, and pump switch were contained within a 30.5 cm × 30.5 cm × 10 cm plastic sealed box with a screened exit for pump air exhaust. The solar panel was

secured to a telescoping aluminium pole and raised above the tree canopy. The cylinder, box, and pole were all secured to an 8.6 cm × 8.6 cm × 244 cm wooden post dug 60 cm into the ground and within the tree line to avoid interference with orchard service equipment. Flow rates of each collection system were measured in the field immediately before and after collections by a compact flowmeter (Gilmont, Barrington, IL).

3.3. Volatile analyses

Upon completion of the volatile emission collection, the Tenax cartridges were sealed and transported to the laboratory for desorption and analyses following published protocols (Beck et al., 2008). A typical volatile analysis included: desorption with diethyl ether (100 ml), concentration of extracted volatile solution to ca. 1 ml via water bath (ca. 40 °C) and Vigreux condenser, and 1 μl injection of desorbed volatiles onto a J&W Scientific (Folsom, CA) DB-Wax column (60 m × 0.32 mm i.d. × 0.25 μm), installed on an HP-6890 gas chromatograph coupled to HP-5973 mass selective detector (Palo Alto, CA). Desorbed volatiles were analyzed via GC–MS using published methods (Beck et al., 2008, 2009). NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. The retention indices (RIs) were calculated using a homologous series of *n*-alkanes on a DB-Wax column. Volatile identifications were verified by injection of authentic samples and comparison to retention times of an internally generated list of volatiles on identical columns. Each experiment was duplicated per site (two collection boxes).

Data from GC–MS analyses were transferred to Microsoft Excel for comparison of retention times and calculation of averages and standard errors. The reported volatile amounts in Table 1are the average of the two collections per experiment ($n = 2$). For inclusion into Table 1, volatiles had to be present and within relatively equal amounts (<20% variation) in both collections. Volatiles were relatively quantified via the following: concentrated samples were adjusted to 2.0 ml with diethyl ether and an aliquot of 250 μl of the volatile sample was combined with 250 μl of an internal standard solution (3 μg ml^{-1}cyclodecanone in ether); samples were analyzed via GC–MS with injections of 1.0 μl at a 1:1 split. Standard calibration curves were obtained using four concentrations of the internal standard over the range of 0.15–30.0 μg ml^{-1} and the results averaged (linear regression analysis, $R^2 = 0.9998$).

3.4. Electroantennogram experiments

The EAG experiments were performed by identical protocols described previously (Beck et al., 2009). The ambient volatiles from the two collections consisted of the concentrated volatiles in diethyl ether (ca. 80 μg) on oven-dried 0.64 cm assay discs and diethyl ether as the NegCtrl discs (Whatman, Sigma–Aldrich, St. Louis, MO). Discs were allowed to air-dry for 5 min, inserted into 14.6 cm Pasteur pipets. The ends of the pipets were temporarily capped with parafilm. Negative control (NegCtrl) discs were prepared using a similar method, but with 10 μl of ether prior to solvent evaporation. The mean female NOW response to the NegCtrl was ca. 80 μV. Positive control (PosCtrl) discs were prepared using the major sex pheromone component (*Z*, *Z*)-11,13-hexadecadienal (50 μg, diluted in pentane, Suterra LLC, Bend, OR). The mean female NOW response to the PosCtrl was ca. 200 μV. The pipets loaded with the volatile bouquets were attached via tubing to a stimulus controller unit (Syntech, Kirchzarten, Germany). The antennae were exposed to each bouquet by a 2-s puff of air and the resulting response recorded. The antennal stimulation was duplicated for each bouquet with a 1 min delay between puffs. NOW antennal responses (μV) were corrected by subtracting the NegCtrl response from the EAG raw response.

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